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Biological characteristics of the strains of *Entamoeba histolytica*
from the carriers in Poznań Palatinate*

Właściwości biologiczne szczepów *Entamoeba histolytica*
u nosicieli w woj. poznańskim

Entamoeba histolytica was described by Lösch in 1875, and, in spite of numerous investigations conducted over the last 85 years, there still exist differences of opinions on its biology, and especially on its life cycle and the host-parasite relationship.

It is generally assumed that *Entamoeba histolytica* is a specific human parasite with a world-wide distribution. The infected persons, however, do not always show any specific diseases symptoms (amoebic dysentery).

There are two trophozoite stages, namely large and small form in the life cycle of *Entamoeba histolytica*. The former is a pathogenic parasite, living in the tissues of the large intestine, while the latter is a commensal form and lives in the lumen of the gut. Although the phenomenon of the transformation of a commensal non-pathogenic organism into a parasitic one is generally known, it is also the cause of two controversial opinions. According to Dobell 1919 *Entamoeba histolytica* is an obligatory pathogen, living in the tissues, and the large form is a permanent stage in the life cycle of the parasite. Dobell denies the occurrence of quite healthy carriers. He assumes that even in apparently healthy carriers there always appear small lesions of the gut wall, which do not cause any outward manifestations of disease. The „*minuta*” is a precystic form, assuring the propagation of the species by encystation.

According to the another opinion formulated first by Kuenen et Swellengrebe 1913, it is the „*minuta*” form that is the basic stage in the life cycle of *Entamoeba histolytica*. According to these authors it is commensal, lives in the lumen of the intestine, and may, under certain conditions, be transformed into the large form and invade the tissues of the host.

According to Hoare 1952 b „although *E. histolytica* is a potentially pathogenic parasite, capable — under certain conditions — of invading the tissues of its host with the production of lesions and clinical symptoms, it does not manifest any evidence of virulence in the majority of carriers,

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in whom the infection is symptomless and the amoebae live in the lumen of the gut as harmless commensals, feeding on bacteria and other faecal contents, without causing any damage to the gut wall".

Neal's 1956 investigations have shown that the virulence of *Entamoeba histolytica* strains primarily depends upon the invasiveness of the protozoon itself.

It has been assumed in Europe that the virulence of *Entamoeba histolytica* is potential, and does not always manifest itself, while the American investigators, and especially Faust 1958 and Craig 1951, classify it among obligatory parasites.

The environmental factors play undoubtedly a considerable role in the development of amoebiasis. Also bacterial flora may create favourable conditions for strengthening the invasion of the "minuta" form into the lumen of the intestine and the host's tissues (Westphal 1938, Phillips et al. 1955, 1958).

According to Brumpt 1925, 1936 there exist two species of *Entamoeba*, which form large ($> 10 \mu$) 4-nucleate cysts, and namely: *Entamoeba dysenteriae* and *Entamoeba dispar*, which are identical morphologically, but are biologically different. The "forma magna" trophozoite of the former is pathogenic and this species may also occur as commensal form in carriers in its "minuta" form. *Entamoeba dysenteriae* is distributed only in warm and tropical regions; *Entamoeba dispar* is a non-pathogenic, commensal species, living in the lumen of the intestine as the "minuta" form exclusively. It is a cosmopolitic species. This division of the *Entamoeba histolytica* into two separate species, i.e. *Entamoeba dysenteriae* and *Entamoeba dispar* basing only on different host reactions has not been generally accepted by zoologists.

Statistical data obtained in Poland from mass examinations during the last years show that about 2 per cent of population are carriers of *Entamoeba histolytica* (Janicki et al. 1950, Gerwel et al. 1954, 1957a, 1957b, Wysocka et al. 1954, Ulewicz 1957). It seems, however, that methods used in mass examinations have not been sensitive enough. Kasprzak et Karlewiczowa 1958 have stated a much higher, and namely 9 per cent of examined persons to be infected with *Entamoeba histolytica*, using Heidenhain's iron-hematoxylin method. 121 children from Children's Homes in Poznań were examined. One can assume that the incidence of *Entamoeba histolytica* in total population of Poland is approximately the same. As has been rightly indicated by Gerwel 1958 "amoebiasis does not constitute one of the major sanitary problems in Poland, as it almost exclusively takes the form of symptomless carrier state. One does very rarely encounter typical cases of amoebiasis, but as a rule they are persons who have stayed in the south of Europe or in the tropics, and there are but a few cases in our literature who might have been caused by the indigenous strains. One can then assume that these strains are as a rule non-pathogenic".

Assuming, according to Hoare that "*Entamoeba histolytica* is a potentially pathogenic parasite" and that both its invasiveness and virulence are the properties of *Entamoeba histolytica* strains themselves (Neal 1956), an attempt has been made to provoke and determine the degree of virulence of the local strains, and to study the morphology of the parasite in different surroundings and its growth in various media.

Material and methods

Six *Entamoeba histolytica* strains from carriers without any outward manifestations and from patients with symptoms of acute amoebic dysentery were investigated. The case histories were as follows:

Cases of acute amoebic dysentery:

Strain K₁ — patient K. I., aged 29, had stayed in Vietnam. Acute symptoms of amoebic dysentery appeared during his way home. Numerous trophozoites (large forms) of *Entamoeba histolytica* have been found in the fresh dysenteric stool, among the necrotic mucosa, mucus and red blood cells.

Strain K₃ — patient K. J., aged 30, had a similar case history.

Cases of symptomless carriers:

Strain W — patient W. M., aged 46, has not travelled abroad. Numerous cysts of *Entamoeba histolytica* and *Endolimax nana* have been discovered in 3-day, refrigerator-stored in + 4°C, normal faeces. The materials obtained from simple sedimentation method were used for inoculation in culture media.

Strain C — patient C. M., aged 60, has not stayed abroad. Single trophozoites (*minuta* forms) as well as cysts of *Entamoeba histolytica* and *Entamoeba coli* trophozoites were found in the fresh, formed faeces.

Strain P — patient P. Z., aged 20, has not been abroad. Some trophozoites (*minuta* forms) and numerous *Entamoeba histolytica*, *Endolimax nana*, *Jodamoeba bütschlii* and *Entamoeba coli* cysts were found in the fresh, formed stool.

Strain M — patient M. H., aged 33, has not stayed abroad. Fresh, loose faeces contained numerous trophozoites (*minuta* forms) and cysts of *Entamoeba histolytica*, as well as trophozoites of *Dientamoeba fragilis*.

In microscopic investigations faeces smears, fixed with Schaudinn's solution and stained using Heidenhain's iron-hematoxylin method, as well as fresh and stained smears from the culture material, and histological preparations from intestine walls of infected guinea pigs were used. In morphological investigations one noted the size of the amoebae, the structure of nuclei, the presence of red blood cells or bacteria in the plasma of the trophozoites, and the presence of glycogen vacuoles, as well as the number and shape of chromatoid bodies in the cysts. Mean size of cysts ($> 10 \mu$), which is the most characteristic and genetic (Burrows 1957, Freedman et Elsdon-Dew 1959) has been considered the main criterion for identification of *Entamoeba histolytica* s. str. (= the large race of *Entamoeba histolytica*). Mean values were calculated basing on at least one hundred measurements.

The inoculations on culture media were made with fresh faeces of a size of a pea grain, taken by a loop when the faeces were formed, or by a pipette, if they were loose. The sole exception was strain W, when the cysts from a decanted, 3-day stool were used.

In the introductory investigations (Kasprzak 1956) a number of experiments were conducted on the growth of *Entamoeba histolytica* strains on the media after Boeck et Drbohlav 1925, Dobell et Laidlaw 1926, Tanabe et Chiba 1928, Cleveland et Sanders (according to Reichenow 1952), Pavlova 1938, and Westphal (according to Reichenow 1952). Basing on those experiments, four best, in our opinion, biphasic media (Boeck et Drbohlav, Tanabe et Chiba, Dobell et Laidlaw, Pavlova) have been chosen for further work, i.e. for isolation and for keeping stock-culture. These media were used in two parallel series, with and without the supplement of Penicillin (1000 units per 1 ml

of medium) added only to the initial test-tubes. All media have been supplemented with rice starch (1-2 loops per one test-tube).

The amoebae were growing in the presence of bacterial flora*.

In order to remove *Blastocystis* sp. from the cultures 1 per cent dextrine was added once (recommended by Reichenow 1952), or the sediment containing the amoebae contaminated with fungi was placed for 2 — 3 hours in the sterile, distilled water in 18°C (Smedley 1956).

The cultures were subinoculated every 48 hours, by transferring about 0.5 ml of the sediment from the original culture to fresh media. The growth of cultures was determined by the number amoebae, discovered in one small power field (ocular 12 ×, objective 6 ×).

Guinea pigs of both sexes from stock, bred in our Laboratory for a number of years, were used for experiments. According to Carrera et Faust 1949 the weight and sex of the animals have no influence on the invasion, but it is recommended to choose animals weighing 300 to 400 g, as such weight facilitates handling. On the other hand Taylor et al. 1950 and Sadun et al. 1950 stated that young, 10 to 25 days old guinea pigs, are more sensitive to infection with *Entamoeba histolytica*. For that reason the animals weighing 180 to 380 g (average weight 280 g, 4 weeks old) were chosen for experiments. Thus, their weight was approximately average of that of the animals used by the authors quoted above.

During the week prior to the operation, as well as during the whole experiment the animals were kept in separate cages, weighed every second day and fed on special vitamin C deficient diet (boiled carrots, potatoes, hay and oat grains). They were not given green vegetables. The application of such a diet was intentional, because ascorbic acid lowers the degree of sensitivity to infection with *Entamoeba histolytica* (Carrera et Faust 1949, Sadun et al. 1951). On the other hand synthetic diet (enriched also with vitamin C) may cause certain changes in the mucosa of the caecum in guinea pigs (Lynch 1957). The diet with both qualitative and quantitative protein deficiency, however, seems to have no major influence on the degree of infection (Carrera, Sadun et Faust 1952). On the day prior to the operation and 24 hours after it the animals were not given any food. Only water was supplied.

The animals were infected using Carrera et Faust 1949 and Taylor et al. 1950 methods. Laparotomy was performed under ether anaesthesia. The inoculum was injected directly into the caecum of the guinea pigs.

The inocula were prepared from 48-hour cultures, grown on Dobell and Laidlaw's medium. They were prepared using the following method: the sediment from several test-tubes, containing starch, amoebae and bacterial flora were collected into the tube containing sterile salt solution and centrifuged for 5 minutes at the speed of 1500 r.p.m. The approximate number of trophozoites per 1 ml of emulsion was estimated by counting the amoebae in a haemocytometer chamber. The volume of the inoculum varied from 1 to

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2 ml, and the count of amoebae from 50 — 100 thousand. The number of amoebae has no influence either on the colonisation in the guinea pigs, or on the character of lesions and the mortality rate of the animals. The injection of 20 thousand amoebae into guinea pig intestine is sufficient to cause dysentery (Carrera et Faust 1949, Taylor et al. 1950, Sadun et al. 1950, Boeck et Mudrow-Reichenow 1955, Krupp et Faust 1959).

The kind of the medium used has no influence on the amoeba's virulence (Neal 1951a).

The animals which lived through the period of 30 days were sacrificed with large doses of ether. Autopsies were performed immediately after sacrifice, and as soon as possible after natural death. All macroscopic changes of alimentary duct and liver were noted, and then the whole large intestine with the neighbouring section of ileum were cut out. The whole was then divided into six parts, according to Carrera et Faust's 1949 scheme. The separate parts were then dissected longitudinally and examined macro- and microscopically (binocular, ocular 12 $\times$, objective 2 $\times$). Fresh microscopic preparations (in NaCl) and preparations fixed with Schaudinn's solution and stained with Heidenhain's iron-hematoxylin were made from the content of separate gut sections and from the material scraped off from the mucosa. Besides, the sections of lesions and suspicious-looking tissues of the gut and liver were fixed in Bouin's solution for histological examination. Some sections were stained with Heidenhain's iron-hematoxylin method, and some with hematoxylin-eosine method, or Van Gieson's method.

During the initial phase of experiments all guinea pigs were examined twice for intestinal protozoa; the first examination was made a week before the laparotomy (coprologic examination), the second — during the autopsy (smears of the intestinal content). As the results of the two examinations were not always identical, coprologic method was discontinued during the further part of the experiments.

The first stage of experiments included isolation of the amoebae from the carriers, their adaptation in culture media, where they were cultivated for 6 months, and the subsequent inoculation of the animals. In the second stage the increase of virulence of the amoebae after their passage through animals was investigated on strains cultivated from the material taken from amoebic ulcers of the guinea pigs. The amoebae were cultivated in vitro till sufficient growth was obtained; this required about 30 days (15 passages).

Results of investigations

The following species of protozoans have been found: *Eimeria caviae* Sheather 1924, *Balantidium caviae* Neiva, da Cunha et Travassos 1917, *Entamoeba cobayae* Walker 1908, *Endolimax caviae* Hegner 1926, *Chilomastix intestinalis* Kuczynski 1924, *Trichomonas* sp., *Enteromonas caviae* Lynch 1922, *Monocercomonoides caviae* (Cunha et Muniz 1921), *Monocercomonas caviae* Grassi 1881. *Chilomastix intestinalis* and *Trichomonas* sp. were the most frequent. Neither *Balantidium*

caviae nor *Eimeria caviae* have been found in the intestine walls of the controls.

It is worth stressing that flagellates were found much more frequently (in 63 per cent) in animals in which amoebiasis did not occur. The animals infected with *Entamoeba histolytica*, on the other hand, showed the presence of flagellates only in 15 per cent of cases.

The penetration of the protozoa derived from spontaneous invasion into the lesions caused by *Entamoeba histolytica* did not take place, with the sole exception of *Balantidium caviae*, which presence in the intestinal walls of three guinea pigs together with *Entamoeba histolytica* was stated. These animals were eliminated from further experiments.

Morphology of amoebae

All the strains used in experiments had been identified as *Entamoeba histolytica* s. str. (i.e., large form of *Entamoeba histolytica*). The size of the cyst of separate strains in the direct faecal smears (JKJ) varied from 12.1 to 13.3 μ , and on fixed and stained preparations from 11.4 to 12.5 μ , respectively (Table I). They contained one to four nuclei with central karyosomes in most (56 — 60 per cent) cases. Chromatoid bodies were almost always rod-shaped with rounded ends, and small, irregularly-shaped bodies were less frequent. Glycogen vacuoles with an indistinct outline appeared in all one-nucleate cysts.

Also "minuta" form trophozoites were found in the faeces of carriers, and 80 — 92 per cent of them contained numerous vacuoles, filled with bacteria and fungi. The dimensions of these trophozoites in fixed preparations varied from 12.7 to 14.5 μ .

Trophozoites — large form — found in the diarrhoeal stool of the two patients, 17.6 to 38.4 μ large (average size 27.3 and 29.0 μ , respectively) contained red blood cells or fragments of tissue-cells of the host in 36 and 44 per cent of cases, respectively. About 10 per cent of large form amoebae contained vacuoles filled with bacteria, instead of red blood cells.

The differences in size of the trophozoites cultivated in vitro were much greater, varying from 9.0 to 30.0 μ . One must, however, stress that large trophozoites, frequently with numerous starch grains, appeared when the growth of the amoebae was abundant. On the contrary, the trophozoites were small in the cultures which growth was not of uniform intensity, or which showed poor growth during the experiment. The variations in the trophozoite size in separate test-tubes were not great, and never of the range of the variations in size of one strain from different passages.

The trophozoites from the cultures in fixed and stained preparations usually contained the nuclei non-typical of the species, with large, eccentric karyosomes and a large peripheral layer of chromatin granules on the nuclear membrane. These non-typical characteristics were appearing as the strain was ageing during the cultivation in vitro.

T a b l e I

Strain	Cysts - direct smears in iodine		Cysts - direct smears, hematoxylin		Trophozoites - large form hematoxylin stained smears	
	size in micra		size in micra		size in micra	
	average	range	average	range	average	Erythrocytes in plasma per cent.
K ₁					27.5	17.6 - 35.8
K ₂					29.0	17.8 - 38.4
W	12.2	10.5 - 14.5	11.4	10.0 - 12.6		36
C	12.1	10.4 - 14.6	11.5	9.9 - 12.8		44
P	12.3	10.2 - 14.8	11.4	9.8 - 12.8		
M	13.3	11.5 - 15.2	12.5	11.0 - 14.8		

Size of cysts and trophozoites (large form) found in the faeces of carriers and patients

T a b l e II

Strain	M a n	Cul- ture	Guinea pig - passage 1				Guinea pig - passage 2			
			Trophozoites		Tissue	Ani- mal No.	Trophozoites		Tissue	Ani- mal No.
			Gut content	Necrotic mucosa			Gut content	Necrotic mucosa		
K ₁	17.6-27.3-35.8	Tropho- zoites: 9.0 - 30.0	7 10.8-14.1-19.0	10.6-13.0-17.3	11.8-14.1-16.5	22	19.7-24.4-33.6	13.6-16.4-20.6	14.1-16.9-19.5	
			7 10.8-14.1-19.0	12.7-15.7-17.8	13.0-16.1-19.1	24	10.8-14.2-18.6	13.6-16.4-21.7	14.1-17.0-22.2	
			12 12.4-14.6-16.8	11.9-14.4-16.8	11.9-15.3-17.9	90	11.6-16.3-21.7	12.5-15.3-16.8	14.7-16.6-18.4	
			28 19.0-24.6-34.7	12.8-15.5-19.0	12.6-15.9-21.7	42	10.8-14.3-20.0	13.9-16.1-21.7	13.0-15.5-17.8	
			30 10.8-15.4-19.3	13.2-15.6-17.8	12.5-15.5-17.3	46	10.8-16.4-24.3	12.4-14.2-16.5	12.1-14.5-16.5	
			66 11.4-18.6-32.5	14.1-17.5-21.7	14.1-17.1-20.6	82	16.7-21.0-26.6	13.8-16.0-21.7	11.9-16.1-19.0	
P	10.0-11.4-12.6*	Varying dimensions:				84	11.4-14.6-22.2	13.8-16.8-21.7	13.3-17.3-21.7	
			36 10.8-14.3-21.7	12.5-14.0-15.7	12.1-14.2-15.7					
			40 10.8-12.7-16.0	11.4-14.3-16.9	11.9-14.3-16.8					
C	9.9-11.5-12.8*		44 10.3-12.3-16.3	11.4-14.8-21.7	13.0-15.8-18.0					

Size of cysts and trophozoites in human faeces, cultures, and in infected guinea pigs (in microns)

Table III

Strains	Virulent strains - Vietnam				Virulent strains - Poznań				Total number of virul. strains Vietnam					Avirulent strains Poznań		Controls	
	K ₁ -Pas.2		K ₃ -Pas.1	K ₃ -Pas.2	W-Pas.1	W-Pas.2	C-Pas.1	C-Pas.2	1	Passage			P	M	Ec	N.o.	
	K ₁ -Pas.1									2	1	2					
Number of examined guinea pigs	10	10	10	9	10	10	7	10	20	19	17	20	6	12	9	8	
Number of guinea pigs with lesions of intestine	4	6	9	9	8	8	6	7	13	15	14	15	0	0	0	0	
Caecum	4	6	9	9	8	8	6	7	13	15	14	15					
Colon asc.	1	2	4	5	3	2		3	5	7	3	5					
Colon tr.	1		3	1	1			1	4	1	1	1					
Colon pasc.		1	3	4				2	3	5		2					
Rectum		2	3	3		2		1	3	5		3					
Ileum																	
Hepar																	
Mean loss of weight	-12/5	-69/24	-59/19	-87/29	-85/30	-90/29	-50/19	-81/26	-35/12	-78/27	-67/24	-85/27					
GP with lesions																	
GP without lesions	+27/11	+6/18	+75/27		+20/8	+13/5	+83/36	-7/2	+51/20	+6/2	+52/21	+3/1	-6/2	+14/6	+28/11	+61/18	
g./per cent. of initial weight	-43/16	-85/28	-109/34	-110/32	-121/35	-84/26	-37/15	-80/26	-76/25	-97/30	-79/26	-82/26					
GP with diarrhoea																	
GP without diarrhoea	-13/05	-59/04	-16/15	-80/26	-84/26	-96/34	-57/22	-85/26	-23/8	-69/24	-60/24	-91/30					
Number of guinea pigs with sympt. of diarrhoea	1	2	2	2	3	4	2	3	3	4	5	7	0	0	0	0	
Number of lethal cases	2	6	6	8	8	8	6	6	8	14	14	14	0	0	0	0	
Mean period of survival (days)	25	18	19	16	14	12	14	14	23	16	15	13					

Summary of experiments on guinea pigs for virulence of six *E. histolytica* strains. Ec-infected with *Entamoeba coli*, N.o. not operated.

Table II shows the list of size of the amoebae from the contents of the large intestine, in the necrotic materials gathered from the surface of the lesions (in the fixed and stained smears) and in the tissues of the gut wall of guinea pigs (histologic preparations). Most of these trophozoites did not contain red blood cells in their plasma. Their size varied considerably; there were both large forms, without red blood cells in the endoplasm, and much smaller amoebae resembling "*minuta*" form, with the endoplasm containing red cells. None of the preparations from the guinea pigs showed trophozoites which average sizes would correspond to those found in the stools of the patients. The trophozoites from the last mentioned source were always 3 — 13 μ larger.

Growth of amoebae in cultures

No particular difficulties have been encountered in isolating and cultivating the amoebae. Dobell and Laidlaw's medium provided the most abundant growth, and, although it must be stressed that there were no major differences in the growth during several passages in the indigenous and Vietnam strains, the latter, however, showed better growth in the first passages. A larger number of trophozoites in the inocula and poor accompanying flora (lack of stool masses) were presumably the cause of this phenomenon. The cultures developed from the material taken from the bottom of caecum ulcers in guinea pigs behaved like Vietnam strains.

The addition of Penicillin to the initial inoculum caused more rapid growth of the amoebae in a few succeeding subcultures, these differences were, however, obliterated in the further passages. Similar observations can be found in the works of Spingarn et Edelman 1952 as well as Norman et Brooke 1955, and others.

Amoebic cultures are usually accompanied by indefinite, mixed bacterial flora. Only in one case (strain W) a monobacterial culture was obtained, which was growing in the presence of *Escherichia coli*, and, in two cases, of two bacteria species: strain K₃ with *Bacillus cereus* and *Pseudomonas* sp., and strain K₁ with *Bacillus cereus* and *Pseudomonas* sp.

In twelve cases the cultures were obtained from the inoculation of the content of caecum lesions from the lethal cases of guinea pigs; four of these cultures were monobacterial ones, where the amoebae were growing either in the presence of *Esch. coli*, or *Bac. cereus*, and in one case in the presence of *Alkaligenes fecalis* and *Pseudomonas* sp.

The growth of monobacterial cultures with *Esch. coli* was excellent. In 90 per cent control tests hundreds of trophozoites, containing numerous starch grains in their protoplasm were discovered during microscopic examination. The growth of the amoebae accompanied by *Bac. cereus*, *Proteus* sp., *Pseudomonas* sp. or *Alkaligenes fecalis* was much poorer, and we succeeded in cultivating them for some months only. Similar results have been described by Cleveland et Sanders 1930, Saito 1950, 1951, Rees et al. 1953, and others.

All the carrier strains were growing in the presence of *Blastocystis*, and neither supplementing the medium with 1 per cent addition of dextrine, nor

washing the cultures with distilled water could remove them. When the latter action was applied the fungus disappeared in the first subcultures, but reappeared again after a few passages.

The fungus disappeared spontaneously in two cultures only, and namely: in strain W after 16 passages, and in strain M after 7 passages. The presence of *Blastocystis* had no influence on the growth of the amoebic cultures.

Blastocystis did not appear in the cultures of Vietnam strains, or in those inoculated with the material from the intestinal lesions in guinea pigs.

Spontaneous encystation of the amoebae could be observed fairly frequently, but mainly in the cultures showing either slow or accelerated growth. The cysts usually had four nuclei, and their size was typical of the species.

Experiments on guinea pigs

109 guinea pigs (51 females and 58 males) were used in the experiments. They were infected with K₁, K₃, W, C, P and M strains of *Entamoeba histolytica*.

The controls were divided into two groups:

I — 8 guinea pigs not operated, which lived in the conditions resembling those of the operated animals.

II — 9 guinea pigs infected by laparotomy with about 100.000 *Entamoeba coli* trophozoites and with the accompanying flora.

Out of 109 guinea pigs infected with *Entamoeba histolytica* seven died during 48 hours following the laparotomy, 5 before 30 days have elapsed for some undefined reasons, and in 3 *Balantidium caviae* was discovered. These animals have not been included into the results.

Four strains out of the six used caused amoebic dysentery in the animals. They were two Vietnam (K₁ and K₃) and two Poznań (W and C) strains. The remaining two indigenous (P and M) strains have not caused any pathological changes whatsoever in any animal infected. Neither were the amoebae discovered in the lumen of the gut.

The most notable symptom in the infected animals was loss of weight, which was the greatest in the animals affected with diarrhoea (the mean loss of weight ranged from 37 to 121 g, i.e., 15 — 35 per cent of the initial weight). In controls the average gains during the 30 days amounted to 61 and 28 g, respectively (18 and 11 per cent of the initial weight). The animals infected with non-virulent P and M strains have either gained nothing, or very little.

Further outward symptoms observed in the infected animals were apathy, anorexia, and their fur lost its gloss and was bristled. In 8 out of 37 guinea pigs inoculated with virulent strains diarrhoea appeared two to three days before death. This symptom was characteristic in animals with extensive ulceration of the colon. Both trophozoites with red blood cells and typical "minuta" forms were discovered in the faeces; more than a half of the latter contained bacteria in their vacuoles.

13 out of 20 guinea pigs inoculated with Vietnam strain showed the lesions of the large intestine and of this number 8 died between the 15th and the 30th days. Similar changes were observed in 14 out of 17 guinea pigs

inoculated with the indigenous virulent strains, and none of them lived longer than 27 days. The average periods of survival for the animals inoculated with Vietnam and indigenous strains lasted 23 and 15 days, respectively (Table III).

The autopsies of the animals infected with *Entamoeba histolytica* have shown: caecum and the adjacent section of ascending colon showed congestion and hyperaemia clearly seen through the serosa. Small lesions with the characteristic red annulus could be seen in the mucosa even macroscopically. Larger lesions were usually of irregular shape, with distinctly proliferated borders, single or multiple, and even stretching on the whole the surface of the caecum mucosa (Phot. 1 — 3). The lesions appeared in the large intestine exclusively. No pathological changes have been found in the end part of small intestine or in the liver. In spite of the apparent lack of liver lesions Rees et al. 1954 succeeded in cultivating *Entamoeba histolytica* from the inoculum of the sections of liver on the culture media.

The lumen of the ulcerated gut levels was filled either with watery masses, or with normal, formed faeces. The watery masses appeared in guinea pigs which showed the symptoms of dysentery.

The vegetative forms of *Entamoeba histolytica* were discovered in the content of the caecum, or even in the ascending colon, but the cysts have never been stated there. The fact of finding cysts of *E. histolytica* in one of the animals is then of particular interest. They were situated in the glandular crypt of the caecum, displaced into the lymphoid tissues of the submucosa (Phot. 4, 5a, and 5b).

The penetration of the amoebae into the gut wall began either from the tip of an interglandular prominence, or, more frequently, from the base of Lieberkühn's crypt (Phot. 6 — 9). The routes of separate amoebae into the intestinal wall generally were not marked by an orifice on the surface of the mucosa. Neither could one notice any particular changes on the routes along which the amoebae were penetrating towards muscularis mucosae. The amoebae were simply found inside a normal reticular tissue, and when they had reached muscularis mucosae they did not penetrate into submucosis, but dispersed in both directions along muscularis mucosae. But even in such states of not very intense penetration of the amoebae into the mucosa a typical signs of inflammation could be seen in submucosis, which was there swollen and infiltrated with numerous cells.

Distinct and sometimes large ulcers on the surface of mucosa were formed when the amoebae penetrated the muscularis mucosae and reached the submucosis. In all these cases Peyer's patches, with channel of invasion opened widely towards the mucosa, were the site of particularly active invasion of the amoebae. It is probable that the amoebae were penetrating through the muscularis mucosae into the patches along the lymph tracts. Bacterial flora always assisted this process, as both the route of penetration of the amoebae from the gut surface and the lymphoid tissues of the submucosa were characterised by advanced destructive processes. The necrotic tissue with few amoebae and leukocytes was extending towards the lumen of the intestine, and it covered the damage of the mucosa like a mushroom with a comparatively thin stem. The necrotic debris was protruding outside and flowing out around the opening of the ulcer, on the surface of healthy mucosa (Phot. 12).

Few amoebae could be found inside the necrotic debris; on its peripheries, on the border of the live tissue, however, they were very numerous (Phot. 10, 11).

Productive processes of both mucosa and submucosis, resulting from destruction processes, which always lead to changes in the intestine wall, caused also considerable displacement of its layers. The fact that the flask-like dilated glandular crypts can be seen extending into the submucosis and even into the Peyer's patches can serve as an example (Phot. 12, 13). One can clearly see the secondary productive changes in such a crypt, e.g., the base of the crypt, through which the amoebae had earlier penetrated towards the lymph tracts and submucosis, is now lined with non-typical, flattened epithelium (Phot. 13).

From the lymph patches the amoebae penetrated still deeper, through tela muscularis into the serosa (Phot. 14, 15). Also in those cases they were presumably moving along the lymph tracts, or through the connective tissue of the muscular layer.

The Vietnam strains of *E. histolytica* which passed through the guinea pigs showed the increase of virulence, which was reflected in the loss of weight of the animals, more extensive ulceration of the gut, involving even the ascending colon, transverse colon and rectum, more frequent cases of diarrhoea and, as a result, shortened period of survival, and higher mortality rate.

The determination of the mean values of the degree of virulence of separate *E. histolytica* strains basing on the changes in the alimentary tract of guinea pigs caused some difficulty. Different authors accept different values and gradation of changes (average caecal scores, average degree of infection). And so, e.g., Neal 1951 took into account the changes in the intestinal wall (gradation 0 — 8) and the consistence of the gut content, Rees et al. 1954 the extension of lesions and the presence of the amoebae in the content of the gut (gradation 1 — 4), Boeck et Mudrow-Reichenow 1955 the changes in the caecum and the presence of amoebae (gradation 0 — 4), and Soloviev 1959, basing on Ščensnovič's 1955 scale, took into account only the changes in the intestinal wall (gradation 1 — 3).

In the present experiment the consistence of the content of large intestine did not always correspond to the pathological changes in the intestinal wall, and the presence of amoebae in the content of the gut could be stated only in cases with lesions.

That is why for the determination of virulence of separate cases the following data were taken into account: number of infected guinea pigs, number of animals with lesions of the large intestine, extension of lesions (their presence in separate levels of large intestine), number of dead animals, the mean time of survival, and the mean loss of weight.

Discussion

All the strains isolated from the carriers represented *Entamoeba histolytica* sensu stricto (corresponding to the large race of *E. histolytica* according to American authors). The size and structure of cysts were considered a basis for determination of the species.

Basing on the result of examinations of the carriers' faeces one can assume that the amoebae were vegetating in the host's organism as commensals. Both the food habits of the trophozoites (Hoare 1952 a), i.e., the food vacuoles filled with bacteria, and the condition of carriers supported this assumption. The nuclei of the majority of trophozoites showed the structure typical of the species, on the other hand one third of the cysts contained the nuclei with eccentric karyosomes. It seems then that the situation of karyosome typical of the species can be taken into account only in vegetative form of the amoeba.

The size of the amoebae in the cultures was constantly varying. The kind of medium and bacterial flora connected with it were here the decisive factor. The investigations of Freedman et Elsdon-Dew 1959 showed, that the mean size of the population of *Entamoeba histolytica* can be varied considerably by a change in the bacterial associates. According to Dobell 1928 the size of the amoebae in cultures or in monkeys depends on the kind of the absorbed food, and the phagocytosis of starch favours the growth of the cells better than the phagocytosis of bacteria. But neither in cultures nor in monkeys do they attain the dimensions of the haematophagous amoebae found in dysenteric patients and kittens. Also in author's cultures the amoebae, which contained numerous starch grains in their plasm, were particularly large.

Also histolytic forms of the amoebae in the unnatural conditions, such as the intestine of a guinea pig, show considerable variations in size, and never reach the size of *E. histolytica* found in dysenteric patients. The size of typical histolytic forms with red blood cells in the endoplasm found in the lumen of the guinea pig gut seldom exceeded 20 μ , so it hardly reached the upper size limit of small forms occurring in man (Brug — op. cit. Hoare 1952 b). On the other hand histolytic forms found in the intestinal walls of guinea pigs never exceeded 20 μ . A similar phenomenon has been described when rabbits were used for experiments (Hunninen et Boone 1957).

In the light of the observations the reports of Bucco et Chieffi 1955 and of Meleney et Zuckerman 1948 about the transformation of small (i.e., *Entamoeba hartmanni*) into the large race after a number of passages, based only on the observation of the growth of the amoebic body size, must cause some doubts. If the size of *Entamoeba histolytica* shows such considerable variations depending on the medium of its growth, one can also assume that a similar dependence may characterise *Entamoeba hartmanni*.

Similarly Rizzotti's 1952 conclusions, suggesting that small race (pseudo-small race; the cysts reaching 11 μ , and the trophozoites 17 μ) was pathogenic, can be considered as doubtful. On the one hand the interpretation of the terms "small race" and "small form" of that author is faulty, on the other he erroneously assumes that histolytic forms in man and in experimental animal are always morphologically identical.

It seems that it is the size of cysts, which should not be less than 10 μ that may be considered a criterion for the determination of *Entamoeba histolytica* "races", i.e., *E. histolytica* and *E. hartmanni*. This value is permanent without regard to the conditions in which the cysts are formed. In the present

experiment both the cysts from the cultures in vitro and those found in the gut of guinea pigs support this opinion.

Many authors and recently also Freedman et Elsdon-Dew 1959 believe that the presence of red blood cells of the host in the endoplasm of invasive form of the amoebae is the typical, and even the only morphological characteristic which permits to distinguish them from commensal form. In connection with this the author's observations may seem of some interest. Among many large forms from the fresh stools of two patients with amoebic dysentery only 36 and 44 per cent, respectively, contained red blood cells in their endoplasm. About 10 per cent of the amoebae showed morphological characteristics of parasitic form, feeding on red blood cells, with one basic difference, and namely their vacuoles contained bacteria instead of red blood cells. They were then not parasitic, but commensal forms. On the other hand histolytic forms, found in the tissues of infected animals, very seldom contain red blood cells. It is generally known that the amoebae found in the human large intestine walls look similar.

It seems then that the traditional term "*forma magna* of *Entamoeba histolytica*" is not strict, as it determines only the size of the amoebae, and does not qualify their biological character. It is probable that large forms of *E. histolytica* can appear in three physiological stages: (a) histolytic invasive form, which invades the host tissues and feeds by means of endosmosis, (b) phagocytic, parasitic form, with red blood cells of the host, found as a rule in the dysenteric stools, and (c) commensal form with bacteria, which undoubtedly may be placed on the border of the two parasitic forms just mentioned, and the small commensal or precystic form.

Already the classical experiment conducted by Hlava 1887 (quoted after Stilwell 1955) who provoked the symptoms of typical dysentery in young cats, introducing the trophozoites of *Entamoeba histolytica* per rectum, proved that the strains from carriers without any outward manifestations of the disease may prove pathogenic for experimental animals. This experiment was later utilised as biological test, used for the identification of amoebic species. Basing on the results of this test, which are always positive in cats, even if commensal forms were used in experiments, an opinion was formed that all amoebic strains, and even those which do not cause any clinical symptoms are virulent. It was, however, stated that this conclusion was too far-fetched, as the cats are particularly sensitive animals and that only cats react to all *Entamoeba histolytica* strains, even to those completely non-pathogenic both for other experimental animals and man (Neal et Vincent 1955).

Further experiments (Meleney et Frye 1933, 1935, quoted after Neal 1957, Josephine 1958) have shown that the degree of susceptibility of cats to separate amoebic strains is different and depends not only on the biological properties of the parasite but also on a number of factors not connected with it, such as individual host resistance, diet, accompanying infections, psycho-physical state of the animal, etc.

Dogs react to the *Entamoeba histolytica* similarly, only the form of the disease is less acute than in cats (Tobie 1940, Thompson et al. 1954).

Rats are the only animals in which amoebiasis causes similar clinical picture as in man (Neal 1951 a, 1951 b, 1957, Neal et Vincent 1955,

Okamoto 1953, Sčensnovič 1955, Soloviev 1959). Like men, these animals do not react to the carrier strains who had had no clinical symptoms, even if the amoebae have been passed through other animals. And though Sčensnovič 1955 succeeded in infecting rats with the strains of carriers who had no manifestation of the disease, they did not cause so severe lesions of the gut as virulent strains. *Entamoeba histolytica* may also in rats pass into small (commensal) form and cysts.

The present observations of guinea pigs infected with virulent, and of those infected with avirulent strains did not always permit to draw conclusions as to the progress of the disease. Even diarrhoea, so characteristic for man and rat infected with amoebae, occurred in guinea pigs only in the most acute cases.

According to Neal 1957 the reaction of guinea pigs to virulent and avirulent strains is similar to that of rats, only dysentery caused by virulent strains is always lethal for the former.

As a result of our investigations one might still add that the state of being a carrier without any outward symptoms of the fact does not occur in guinea pig, i.e. *Entamoeba histolytica* does not reach the cyst phase, notwithstanding the infection having been caused by virulent or avirulent strains. Loss of weight was the only noticeable symptom in guinea pigs infected with avirulent strains. The occurring of the cysts in one of the guinea pig should be considered as exceptional. In that respect we share the opinion of Taylor et al. 1950 and Stam 1958.

It was not stated that the susceptibility of guinea pigs to the infection with *Entamoeba histolytica* depended on the specific parasitic fauna of the animals. It was, on the other hand, stated that amoebic lesions may be used by *Balantidium caviae* as the gate of invading the gut wall. In the controls these *Ciliata* have been found only in the lumen of the intestine.

One could also notice a certain biological dependence of *Entamoeba histolytica* and flagellates, and namely the flagellate fauna was always poor in the guinea pigs infected with *E. histolytica*. Krupp et Faust 1959 have observed a similar phenomenon.

Also other internal and external factors such as, e.g., bacterial flora are undoubtedly not without importance for *E. histolytica*. One can, however accept Phillip's et al. 1955, 1958 and Taylor's et al. 1959 conclusions that bacterial flora may be an important synergic factor for amoebae, the virulence, however, of separate strains is the exponent of biological characteristics of the amoebae themselves (Neal 1956, Neal et Vincent 1956).

Comparing the degree of virulence of the indigenous and Vietnam strains we took into account not only the intensity of typical changes (lesions of the intestine walls) which, according to many authors (Neal, Rees et al., Sčensnovič, Rogova, Soloviev, Boeck et Mudrow-Reichenow a.o.) are supposed to be the main manifestation of virulence, but also other factors which are important in the author's opinion (such as loss of weight, mean period of survival, the number of lethal cases, etc.). If one based only on the picture of autopsy one might estimate the Vietnam strains as more virulent. Both the survival period, however, and the number of lethal cases indicate that indigenous strains proved rather more virulent

for guinea pigs, than Vietnam ones. Moreover, indigenous strains were as highly virulent already in the first passage as in the second, while the virulence of Vietnam strains in the second passage was much higher than in the first, not higher, however, than that of indigenous strains.

The results of our experiments seem to confirm Neal's 1957 and Hoare's 1958 suggestions about the possibility of existence of two types of *E. histolytica* s. str. (i.e., the large race of *E. histolytica*) which are morphologically identical, but the virulence and distribution of which are different. While the avirulent type is widely distributed all over the world (*E. dispar*, according to Brumpt 1925), the virulent one is found as a rule in warm and tropical countries (*E. dysenteriae*, according to Brumpt 1925). The incidence of highly virulent strains of *E. histolytica* among our population indicates not only the possibility of these strains being transferred to the regions with cooler climate, but it also suggests, contrary to Hoare's 1958 opinion, the possibility of complete accommodation of these strains to our conditions.

Summary

The investigations were conducted on the strains of *Entamoeba histolytica* sensu stricto (i.e. the large race of *E. histolytica*). Basing on the results of examinations of the carrier's faeces one can assume that the amoebae occurred in the host organisms as commensals. This was indicated both by food habits of the amoebae and the general condition of the hosts.

In unnatural conditions, such as cultures in vitro and non-specific host (guinea pig), one can observe considerable deviations in trophozoite structure. The cysts of *E. histolytica*, on the other hand, retain their typical size, without regard to the conditions in which they are formed.

All the strains of *E. histolytica* notwithstanding their origin, behaved identically in the cultures; their growth depended directly on the kind of medium and the flora connected with it. *Blastocystis* sp., which often accompanies the cultures, does not change their growth.

Amoebic dysentery is not always lethal for guinea pigs, even if the strains which had caused it are distinctly pathogenic to man. On the other hand the amoebae do not form cysts in guinea pigs.

Two types, virulent and avirulent, have been found among the indigenous strains isolated from the carriers. The virulence of indigenous strains to guinea pigs is equal to that of the strains decidedly pathogenic to man, occurring in tropical countries.

It seems that the large form of *E. histolytica* (the traditional "forma magna") can be found in different physiologic forms: (1) as histolytic form found as a rule in the host tissues and fed by osmosis connected with extra-cellular digestion (histolysis), (2) as parasitic form fed by phagocytosis of red blood cells of the host, which can be found as a rule in the dysenteric stools, and (3) as commensal form, fed on bacteria, which constitutes the intermediate form between parasitic and small, commensal, or pre-cystic forms.

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STRESZCZENIE

Badania przeprowadzono nad szczepami *Entamoeba histolytica* s. stricto (odpowiednik dużej rasy *E. histolytica* autorów amerykańskich). Cztery szczepy izolowano z kału bezobjawowych nosicieli (szczepy rodzime), a dwa z kału chorych, z objawami ostrej pełzakowicy (szczepy wietnamskie). Jako podłoża sztuczne stosowano cztery pożywki dwufazowe. Jako zwierząt doświadczalnych użyto 109 świnek morskich, które zarażano metodą Carrery i Fausta.

Najważniejsze wyniki obserwacji przedstawiają się następująco:

Czerwonka pełzakowa, nawet gdy wywołując ją szczepy *Entamoeba histolytica* są dla człowieka zdecydowanie chorobotwórcze, jest dla świnek

morskich nie zawsze śmiertelna. Z drugiej strony pełzaki u świnek morskich nie przeistaczają się w postacię przetrwalnikowe.

Wśród szczepów rodzimych *E. histolytica*, wyodrębnionych od nosicieli, spotykamy dwa typy szczepów: wirulentne i awirulentne. Wirulencja szczepów rodzimych w stosunku do świnek morskich jest równa wirulencji szczepów dla człowieka zdecydowanie chorobotwórczych, występujących w krajach tropikalnych.

Wydaje się, że postać duża *E. histolytica* (tradycyjna „forma magna”) występuje w różnych postaciach fizjologicznych: jako postać histolityczna, znajdująca z reguły w tkankach żywiciela, a odżywiająca się na drodze osmotycznej poprzez trawienie zewnątrzkomórkowe (histoliza), postać pasożytnicza odżywiająca się przez fagocytowanie czerwonych krwinek żywiciela, występująca z reguły w wypróżnieniach czerwonych, oraz postać komensalna, fagocytująca bakterie, która stanowi formę przejściową między postaciami pasożytniczymi a postacią małą komensalną lub precystalną.

EXPLANATION OF PLATES I—VI

- Phot. 1. Caecum. One large and two small dysenteric lesions situated in ileo-caecal region. X 1.2. (Strain W).
- Phot. 2. Caecum. Small, separate ulcerations strewn all over the mucosa. X 3.5. (Strain C).
- Phot. 3. Caecum. Extensive ulceration of the mucosa. X 1.5. (Strain C).
- Phot. 4. Caecum. Deep ulceration of submucosa in which the amoebic cysts are seen. About X 70. (Strain C).
- Phot. 5a and 5b. Fragments of Phot. 4. Mononucleate cysts with chromatoid bodies in the crypt extended into lymphoid tissue of submucosa. About X 1200.
- Phot. 6. Ascending colon. Intestinal wall without ulceration. Single amoebae are invading the tissue from the tip of an interglandular prominence of the mucosa. About X 150. (Strain W).
- Phot. 7. Fragment of Phot. 6. Amoeba penetrating through the cells of mucosal epithelium into intestinal wall. About X 2000. (Strain W).
- Phot. 8. Caecum. Ulcerated intestinal mucosa, necrotic debris stretching on the mucosa surface. Pronounced oedema and infiltration of submucosis. X 120. (Strain K₃).
- Phot. 9. Fragment of Phot. 8. Amoebae in destroyed glandular crypts and at the base of a strongly dilated one — the gate of penetration of the deeper layers of intestinal wall. X 450. (Strain K₃).
- Phot. 10. Ascending colon. Superficial lesions of mucosa. Extensive areas of necrosis are evident, in which single amoebae are seen. Crypts partly destroyed and filled with numerous amoebae. X 120. (Strain K₃).
- Phot. 11. Fragment of Phot. 10. Numerous amoebae forming compact colonies in destroyed mucosa, reaching the muscularis mucosae, X 740. (Strain K₃).
- Phot. 12. Ascending colon. Ulcerations reaching submucosis, necrotic debris around the crater of the ulcer. Two bottle-like dilated crypts displaced into lymphoid tissue of submucosa. X 50. (Strain K₃GP).
- Phot. 13. Caecum. Dilated bottle-shaped crypt extending into lymphoid tissue of submucosa. The bottom of the crypt lined with flattened epithelium, single amoebae are seen inside the crypt. X 250. (Strain W).
- Phot. 14. Caecum. Ulceration of mucosa over the Peyer's patch. Numerous amoebae are seen in the mucosal crypts, and some in the lymphoid tissue and submucosis. X 130. (Strain WGP).
- Phot. 15. Fragment of Phot. 14. Amoebae grouped beneath the muscularis propria, close to the serosal layer. About X 1000. (Strain WGP).



